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Heat Shock Protein 90 Homeostasis Controls Stage Differentiation in *Leishmania donovani*

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The differentiation of *Leishmania* parasites from the insect stage, the promastigote, toward the pathogenic mammalian stage, the amastigote, is triggered primarily by the rise in ambient temperature encountered during the insect-to-mammal transmission. We show here that inactivation of heat shock protein (Hsp) 90, with the use of the drugs geldanamycin or radicicol, mimics transmission and induces the differentiation from the promastigote to the amastigote stage. Geldanamycin also induces a growth arrest of cultured promastigotes that can be forestalled by overexpression of the cytoplasmic Hsp90. Moreover, we demonstrate that Hsp90 serves as a feedback inhibitor of the cellular heat shock response in *Leishmania*. Our results are consistent with Hsp90 homeostasis serving as cellular thermometer for these primitive eukaryotes, controlling both the heat shock response and morphological differentiation.

INTRODUCTION

Protozoan parasites of the genus *Leishmania* are transmitted by blood-feeding sand flies to mammalian hosts, including humans, whereby they encounter a rise in ambient temperature. Subjecting cultured insect stages, the promastigotes, of various *Leishmania* species to a similar temperature upshift in vitro results in the transient posttranscriptional upregulation of both heat shock protein (Hsp) 90 (also known as Hsp83) and Hsp70 synthesis and a persistent induction of Hsp100 expression (Argaman *et al.*, 1994; Brandau *et al.*, 1995; Krobitsch *et al.*, 1998). Moreover, species such as *L. mexicana*, *L. pifanoi*, and *L. donovani* will, during heat stress and acidification of the medium, show a morphological transition toward the mammalian stage, the amastigote (Zilberstein and Shapira, 1994; Saar *et al.*, 1998). Such axenic amastigotes are indistinguishable from true host tissue-derived intracellular amastigotes (Bates, 1993; Pan *et al.*, 1993; Charest and Matlashewski, 1994; Charest *et al.*, 1996; Gupta *et al.*, 1996; Saar *et al.*, 1998). Thus, the rise of temperature encountered during transmission can be viewed not as stress but rather as signal for cellular differentiation.

We have shown previously that Hsp100 is critical for the expression of some amastigote stage-specific proteins and for overall virulence of the parasites (Hubel *et al.*, 1997; Krobitsch *et al.*, 1998); however, gene replacement mutants lacking Hsp100 still show, induced by elevated temperature, morphological differentiation to viable amastigote-like culture stages, indicating that the induction of morphological differentiation occurs independently or upstream of Hsp100 (Krobitsch *et al.*, 1998; Krobitsch and Clos, 1999).

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Another chaperone, Hsp90 (Hsp83), is one of the most abundant proteins in *Leishmania*, constituting 2.8% of the cellular protein (Brandau *et al.*, 1995). Hsp90 (Hsp83) is encoded by multiple gene copies and is found in the soluble fraction of the cytoplasm (Shapira and Pinelli, 1989; Brandau *et al.*, 1995; Hubel and Clos, 1996). Its sequence is distinct from the Grp94 homologue of *L. infantum*, which was characterized recently (Larreta *et al.*, 2000). In higher eukaryotes and in yeast, cytosolic Hsp90 has been known to interact directly with ligand-dependent transcription factors and cell cycle regulators (Rutherford and Zuker, 1994; Scheibel and Buchner, 1998; Buchner, 1999). Its interaction with regulatory factors is a prerequisite for such factors to interact properly with ligands and target molecules. In addition to the typically dimeric Hsp90, the functional complexes usually include other heat shock proteins and associated factors (Chang *et al.*, 1997; Scheibel and Buchner, 1998). The impact of Hsp90 on the morphological differentiation of multicellular organisms was demonstrated recently, and a role as molecular capacitor of morphological evolution has been proposed (Rutherford and Lindquist, 1998; Lele *et al.*, 1999). In addition, Hsp90 can also play a more general chaperoning role maintaining the structure of heat-labile proteins (Nathan *et al.*, 1997; Scheibel *et al.*, 1998). Although viable Hsp90 null mutants could not be obtained so far, inactivation of Hsp90 by highly specific inhibitors such as geldanamycin (GA) or radicicol (RAD) (Whitesell *et al.*, 1994, 1998; Schulte *et al.*, 1998) has been helpful in assessing the impact of this Hsp family on various cellular processes (Uma *et al.*, 1997; Ali *et al.*, 1998; Galigniana *et al.*, 1998; Rutherford and Lindquist, 1998; Zou *et al.*, 1998; Lele *et al.*, 1999; Morano and Thiele, 1999; Nathan *et al.*, 1999; Yorgin *et al.*, 2000).

In this article we present evidence that Hsp90 (Hsp83) of *L. donovani* plays a key role in the progression of this parasite from its insect stage toward the pathogenic mammalian stage. We also show an involvement of Hsp90 in cell cycle control and cellular stress response.

MATERIALS AND METHODS

Parasite Strains and Culture

In our analyses we used the Lo8 strain of *L. donovani*, a gift from D. Zilberstein (Techniow, Haifa, Israel).

Promastigotes frozen directly after a passage through BALB/c mice were thawed and cultivated for up to 2 mo at 25°C in M199 medium supplemented with 25% fetal calf serum and 20 μg/ml gentamicin. For in vitro differentiation into the amastigote stage, cells were treated as described (Krobitsch *et al.*, 1998). Briefly, cells were heat shocked for 24 h at 37°C and then cultivated for 5 d at 37°C in mildly acidic medium (pH 5.5).

Cell density was determined with the use of a CASY cell counter (Schärfe Systems).

To select for GA escape mutants, promastigotes were cultivated for several weeks under 50 ng/ml GA (Sigma, St. Louis, MO; dissolved in 100% DMSO). When the culture resumed growth, the GA concentration was increased in 50 ng/ml steps up to 150 and 200 ng/ml.

Electrotransfection

Electrotransfection was performed essentially as described (Laban and Wirth, 1989; Kapler *et al.*, 1990; Krobitsch *et al.*, 1998).

Indirect Immunofluorescence Microscopy

Leishmania cells (1×10^7) were harvested by centrifugation, resuspended in PBS, and applied to glass slides coated with poly-L-lysine. After fixing the cells for 5 min in methanol, the slides were incubated with blocking buffer (0.2% iBlock, 0.02% Tween 20, 0.1% Triton X-100 in PBS). Slides were incubated for 1 h at 35°C with a monoclonal anti–alpha-tubulin antibody (clone B-5–2-1; Sigma) 1:2000 in blocking buffer, followed by incubation with goat anti-mouse antibody, coupled with dichlorotriazin amino fluorescein (DTAF) (1:50; Dianova, Hamburg, Germany).

The samples were analyzed on a Leica DM RB microscope with a confocal TCS NT system. Fluorescence was measured in the FITC channel.

Scanning Electron Microscopy

Leishmania cells were washed twice in PBS, fixed in 2% glutaraldehyde in sodium cacodylate buffer, and post-fixed with 1% osmium. Samples were dehydrated at increasing ethanol concentrations (30–100%). After critical point drying, samples were treated with gold and analyzed on a Philips SEM 500 electron microscope.

Immunoblot Analysis

SDS-PAGE and Western transfer were performed as described (Brandau *et al.*, 1995; Hubel *et al.*, 1995). Briefly, membranes were treated with blocking buffer (5% milk powder and 0.1% Tween 20 in Tris-buffered saline), with antibodies (1:2500–1:5000 in blocking buffer) against *Leishmania* heat shock proteins (Hubel *et al.*, 1995), and with anti-chicken IgG–alkaline phosphatase conjugate (Dianova; 1:2500 in blocking buffer). Anti-A2 mAb, a gift from Greg Matlashewski (McGill University, Montreal, Quebec), was used at a 1:50 dilution; the secondary AP-conjugated goat anti-mouse IgG was used at 1:2500 dilution. Blots were stained with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Pulsed Field Gel Electrophoresis

Leishmania cells were harvested by centrifugation and washed twice in PBS. After centrifugation, parasites were resuspended in PBS and mixed with an equal volume of prewarmed 1% InCert agarose (FMC BioProducts, Rockford, ME). The mixture was aliquoted in block formers (Bio-Rad, Munich, Germany) at 2.5×10^7 parasites per block. For cell lysis, the agarose blocks were incubated for 2 d at 37°C in 0.5 M EDTA, pH 9.0, and 1% Laurylsarcosin containing 2 mg/ml Proteinase K.

Pulsed field gel electrophoresis was performed at 13°C in 0.25 × TBE electrophoresis buffer in a Rotaphor unit (Biometra, Goettingen, Germany) under the following conditions: interval in sec 100 → 10 log, switching angle 120 → 110 lin, voltage 200 → 150 log. Chromosomes from *Saccharomyces cerevisiae* strain YPH80 (New England Biolabs, Beverly, MA) served as size standards.

After staining with ethidium bromide (1 μg/ml), the gel was blotted onto a positively charged nylon membrane (Qiagen, Hilden, Germany) by alkaline transfer (Sambrook *et al.*, 1989). The membrane was hybridized with a digoxigenin-labeled Hsp90 gene probe (DIG DNA Labeling and Detection Kit, Roche Molecular Biochemicals, Mannheim, Germany). The probe was detected with the use of anti-digoxigenin–AP Fab fragments (1:2000; Roche) in PBS with 0.2% iBlock (Tropix, Bedford, MA) and 0.02% Tween 20. Blots were stained as described above.

Flow Cytometric Analysis

Leishmania parasites were cultivated for 24 h at 37°C or at 25°C with or without 100 ng/ml GA. Log phase parasites had a cell density of $\sim 5 \times 10^6$ cells/ml; stationary phase cells had a cell density of $\sim 7 \times 10^7$ cells/ml.

For analysis, 4×10^6 parasites were harvested by mild centrifugation, washed twice in PBS, and fixed for 1 h in 1 ml PBS containing 70% methanol, at 4°C. After the methanol was removed and the cells were resuspended in PBS containing 20 μg/ml RNase A (20 min, 37°C), the cells were harvested, resuspended in citrate buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, pH 7.0, 0.1% Triton X-100), and labeled for 20 min with 1 μM SYTOX Green nucleic acid stain (Molecular Probes, Eugene, OR). After staining, cells were washed two times in PBS supplemented with 5% fetal calf serum and 0.01% sodium azide. Samples were stored in the dark at 4°C until analysis. Fluorescence was monitored by flow cytometry on a fluorescence-activated cell sorter (Becton Dickinson, Heidelberg, Germany) counting 10,000 cells per sample.

Imaging

Halftone images were digitalized on a flatbed scanner (Agfa Snapscan 600, Agfa-Gevaert, Leverkusen, Germany) or a Nikon Coolscan (Duesseldorf, Germany) 35 mm film scanner. Images were cropped and optimized for color saturation with the use of Adobe Photoshop software, version 5.0. Images were combined with line drawings and text with the use of Claris Draw (Claris Corp., Santa Clara, CA) software, version 1.0d.

RESULTS

Geldanamycin Treatment Induces Growth Arrest in G2 Phase of the Cell Cycle

Treatment of *L. donovani* promastigotes (Ld wt) with geldanamycin results in a dose-dependent growth arrest (Figure 1A). To determine whether this effect is due to the inhibition of the cytoplasmic Hsp 90 species, we transfected *L. donovani* cells (Ld wt) stably with a cosmid, pcos90, derived from an *L. donovani* genomic DNA library. This cosmid includes a part of the large multicopy Hsp90 gene cluster that encodes the cytoplasmic Hsp90. Cells

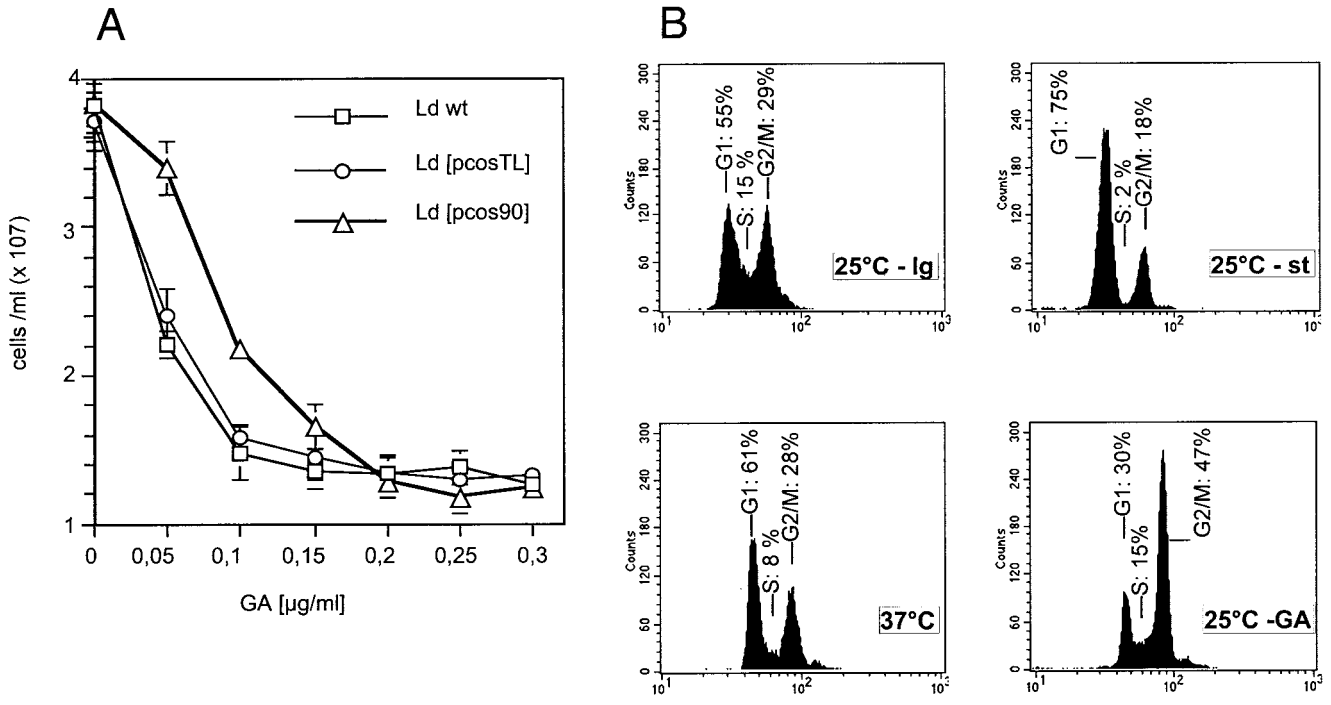

Figure 1. Geldanamycin-induced growth arrest. (A) Cells of three strains, wild-type (Ld wt), vector control (Ld [pcosTL]), and Hsp90-overexpressing cells (Ld [pcos90]) were seeded at 5×10^6 cells/ml and incubated for 24 h at the indicated concentrations of geldanamycin (GA). Cell density was measured, and the mean values from six independent experiments were calculated and plotted against drug concentration. The error bars represent the SD. (B) *Leishmania* promastigotes from logarithmically growing (25°C - lg) or from stationary (25°C - st) culture as well as cells heat shocked at 37°C or treated with GA (200 μg/ml) were analyzed by flow cytometry. Culture aliquots were removed after 24 h and stained with SYTOX. G1, S, and G2/M refer to the corresponding cell cycle stages. Percentages of cells in respective cell cycle stages are given.

transfected with the cosmid vector, Ld [pcosTL] (Kelly *et al*., 1994), served as control. Quantification of Hsp90 expression in the recombinant strains revealed a 35% overexpression of Hsp90 in *L. donovani* Ld [pcos90] compared with both Ld wt and the vector control strain, Ld [pcosTL] (Wiesgigl, unpublished data). This modest increase of Hsp90 expression is due to the high number of *Hsp90* genes, which form large tandem clusters (Hubel and Clos, 1996). Wild-type promastigotes and both recombinant strains were then tested for growth under various GA concentrations. As shown in Figure 1A, we observed a reduced growth rate for Ld wt and Ld [pcosTL] cells at a concentration of 50 ng/ml GA, whereas 100 ng/ml leads to a growth arrest. The monitored increase of cell density from 5×10^6 to ~1–1.5 $\times 10^7$ cells/ml indicates that cells are arrested after one complete cell cycle. In contrast, the Ld [pcos90] cells that overexpress Hsp90 show a half-maximal growth rate at 100 ng/ml GA, whereas a total arrest requires 150 ng/ml, in good correlation with the 35% increase of cytoplasmic Hsp90. This indicates that GA acts specifically on Hsp90, because overexpression of this protein can forestall the effect of the drug.

Given the known number of Hsp90 molecules per cell (1.5×10^6) (Brandau *et al*., 1995) and the cell density (5×10^6/ml), we calculate Hsp90 concentration as 12.5 pmol/ml. Opposed to that, 50 ng/ml of GA, 89 pmol/ml, are required to obtain a half-maximal growth arrest. The nominal seven-fold molar excess of GA over Hsp90 that is sufficient for Hsp90 inhibition is probably even lower in reality given that GA is unstable in aqueous solution. This would indicate a free diffusion of GA through the cell membranes of *Leishmania* promastigotes and a near-stoichiometrical mode of interaction with Hsp90.

Withdrawal of geldanamycin will, over time, reverse the growth arrest; however, up to 1 wk is required before cell division resumes.

To compare the GA-induced growth arrest with heat shock-induced or stationary phase growth arrest, we performed a flow cytometric analysis with SYTOX-stained cells (Figure 1B). Cells growing at logarithmic rate (25°C - lg) show an even distribution between G1-phase and G2/M-phase and a considerable S-phase signal. Promastigotes in stationary phase (25°C - st) are arrested primarily in the G1 phase of the cell cycle. Heat-shocked cells (37°C) are distinguished by a reduced S-phase signal. In contrast, geldanamycin-treated cells (25°C - GA) undergo an arrest that appears to occur primarily in the G2/M-phase of the cell cycle. The effect of the drug, therefore, differs from that of heat shock or high cell density in stationary phase. Under heat shock and under GA treatment, there is a general shift of the populations toward an increased fluorescence. This shift is observed consistently and may reflect an increased autofluorescence that we observe under these conditions (Wiesgigl, unpublished observations).

Continuous Culture of L. donovani *Cells under GA Results in Spontaneous Amplification of the Hsp90 Gene Locus*

The previous experiment showed that cells which overexpress Hsp90 are partially resistant to GA. We wanted to know whether *Leishmania* cells cultivated at threshold concentration are able to develop spontaneous escape mechanisms. To this end we started a prolonged cultivation of promastigotes beginning at 50 ng/ml of GA. As soon as these cells resumed cellular growth, the concentration was increased gradually up to 150 and 200 ng/ml (corresponding to escape populations I and II), respectively. SDS-PAGE analysis of wild-type cells showed a normal protein pattern after Coomassie blue staining (Figure 2A). In contrast, cell lysates of escape strain cells revealed a single strongly induced protein band in the 90-kDa range, whereas the rest of the protein pattern remained unchanged. Immunoblot analysis with the use of specific anti-HSP antibodies revealed the prominent band to represent cytoplasmic Hsp90 (Figure 2B). Scanning densitometric analyses revealed an 8- to 16-fold increase of Hsp90 levels for the escape strains compared with wild type. At 270 pmol/ml of GA and 200 pmol/ml of Hsp90 in the escape mutants, the nominal molar ratio (Hsp90:GA) is only 1:1.35. Given that GA has only limited stability, Hsp90 is probably in molar excess over geldanamycin.

Spontaneous gene amplification is known to occur in *Leishmania* and was identified as a mechanism for developing drug resistance (Beverley, 1991; Segovia, 1994; Haimeur and Ouellette, 1998). To test whether *Hsp90* genes were amplified in the escape populations, we separated chromosomes of wild-type and escape mutant cells by pulsed-field gel electrophoresis. Figure 2C displays the chromosome pattern of *L. donovani* and of the two escape populations after ethidium bromide staining. The gel was then subjected to a Southern blot, and the membrane was probed with a 750-bp fragment derived from the ORF of *Hsp90* (Figure 2D). The uppermost arrow points at the signal obtained from the chromosome that harbors the *Hsp90* gene cluster (Hubel and Clos, 1996). No increase is observed with the escape populations; however, additional bands (arrows) are observed in the karyotype of the escape mutant populations that are absent from the wild-type pattern. We conclude that inhibition of Hsp90 by GA is bypassed by a spontaneous episomal amplification of the *Hsp90* gene cluster encoding the cytosolic Hsp90 or of parts thereof. Indeed, the escape population shows a remarkably different dose response (Figure 2E). Growth of escape mutants is reduced by 50% compared with wild-type control in the absence of GA. The IC_{50}, however, was determined as 525 ng/ml of GA, as opposed to 32 ng/ml of GA determined as IC_{50} for wild-type *L. donovani*. Thus, the tremendous overexpression of Hsp90 slows growth by 50% but increases GA tolerance by a factor of 16.

Because Hsp90 is usually organized in a multiprotein complex (Scheibel and Buchner, 1998), we tested whether overexpression of other proteins could also bypass the GA-induced growth arrest. Such proteins could be client proteins, chaperone complex partners, or, for that matter, other Hsp90 family members such as Grp94. To test this possibility, we performed a genetic complementation screen. *L. donovani* promastigotes were transfected with a *L. donovani* genomic DNA cosmid library and placed under selection with GA or under double selection with G418 and GA. Cosmid DNA from proliferating transfectants was isolated and used to transform *Escherichia coli*. Cosmid clones were then analyzed by PCR or Southern Blot for the presence of *Hsp90* gene copies. All 181 clones that conferred GA resistance to *L. donovani* harbored *Hsp90* gene copies that encode the cytosolic form of Hsp90. This is further indication of the specificity of GA for Hsp90 and the importance of this chaperone for proliferation of promastigote *Leishmania* cells. It also excludes the possibility that the growth arrest induced by geldanamycin is due to an interaction with other Hsp90 family members.

Hsp90 as Regulator of the Stress Response in Leishmania

In higher eukaryotes, activation of heat shock genes in response to stress is mediated by heat shock transcription factors of the HSF-1 type (Morimoto *et al*., 1994; Morimoto and Santoro, 1998). Recent results show that components of the Hsp90 chaperone complex may be involved in regulation of HSF-1 activity (Ali *et al.*, 1998; Zou *et al*., 1998). In *Leishmania*, there is no evidence for the existence of either heat shock promotor elements or heat shock transcription factor(s). Elevated temperature alone will induce Hsp synthesis, which is regulated at a posttranscriptional level. Common chemical inducers of the cellular stress response do not induce heat shock protein synthesis in *L. donovani*, probably because of the lack of transcription regulation (Brandau *et al.*, 1995; Clos *et al*., 1998).

Surprisingly, the inhibition of Hsp90 in *Leishmania* parasites with the use of GA still induces overall Hsp synthesis that, in turn, leads to increased Hsp100 concentrations. Figure 3 shows an immunoblot of cell lysates, derived from promastigotes treated with various GA concentrations. The filter was probed with antibodies directed against *Leishmania* Hsp70, Hsp90, and Hsp100. Although Hsp100 is barely detectable in control cells, GA induces a dose-dependent increase of Hsp100 levels. We observe no significant increase of either Hsp70 or Hsp90. Both heat shock proteins are highly abundant in unstressed cells and do not increase under heat stress either (Brandau *et al*., 1995). Nevertheless, metabolic labeling experiments with the use of ^{35}S-methionine revealed both Hsp70 and Hsp90 to be synthesized at increased rates under GA treatment (Wiesgigl, unpublished data). Hsp90 homeostasis obviously plays some role in the posttranscriptional control of heat shock protein synthesis.

Geldanamycin Treatment Induces Amastigote-specific Protein Synthesis

The similarity of the effects of heat shock and GA treatment prompted us to compare the two stimuli with regard to amastigote-specific gene expression. Promastigote-to-amastigote differentiation can be induced in vitro by the application of a heat shock for 24 h and subsequent acidification of the culture medium. The synthesis of a set of closely related proteins, the A2 protein family (Charest and Matlashewski, 1994; Charest *et al*., 1996), is a hallmark for this stage differentiation process. These proteins with so far unknown functions are important for the virulence of the parasites and can be detected both in axenically cultured and in tissue-derived amastigotes, but not in promastigotes.

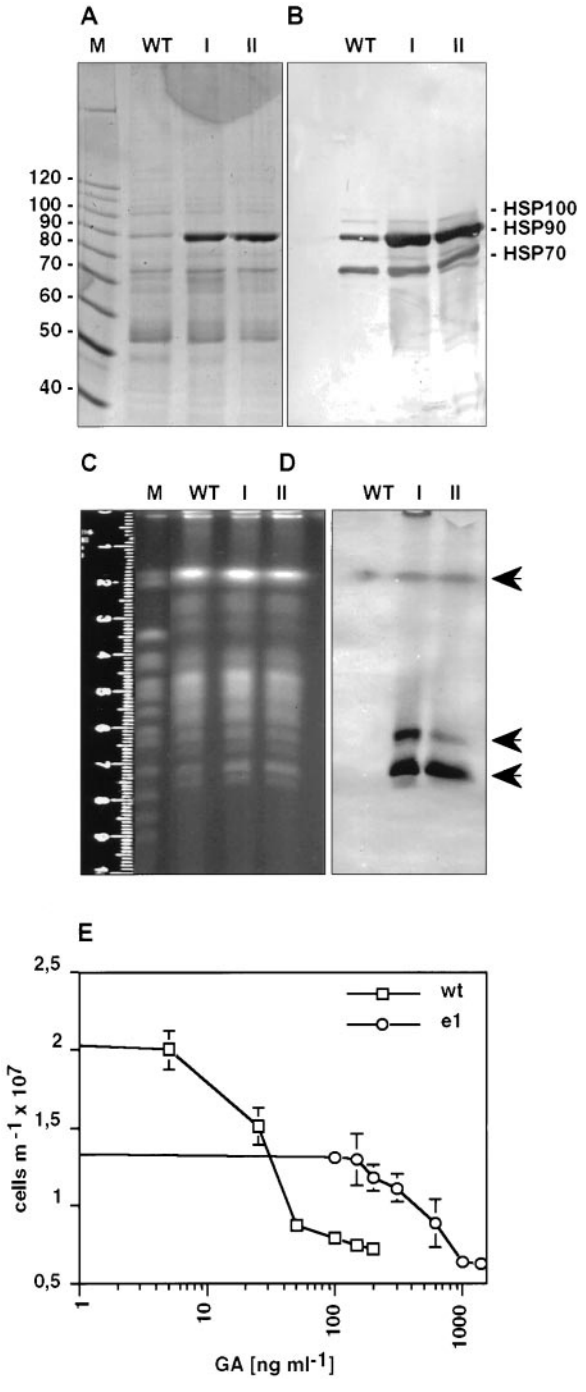

Figure 2. Amplification of Hsp90 gene copies attributable to geldanamycin selection. (A) SDS-PAGE analysis of wild-type promastigotes (WT) and of two escape strains, I and II, selected at 150 and 200 ng/ml GA, respectively. An equivalent of 10^6 cells per lane was dissolved in sample buffer and run on a 7.5% gel. The gel was stained with Coomassie blue. The sizes of selected marker proteins (10-kDa ladder; Life Technologies, Gaithersburg, MD) are shown on the left. (B) Immunoblot analysis of the samples described in A. After Western blot, the membrane was probed with a mixture of antibodies directed against Hsp70, Hsp90 and Hsp100. (C) Karyotyping. Chromosomes of *L. donovani* wild-type and escape strains I and II were separated by pulsed-field gel electrophoresis and stained with ethidium bromide. *Saccharomyces cerevisiae* chromosomes (strain YPH80) were used as size markers (M). (D) The gel shown in C was subjected to Southern blot, and the membrane was hybridized with a digoxigenin-labeled *hsp90* gene probe. The arrows point at bands positive for Hsp90 hybridization. (E) *L. donovani* wild-type (wt) or escape mutant (e1) cells were seeded at 2×10^6 and incubated for 24 h at increasing GA concentrations. Cell density was measured and plotted against GA concentration.

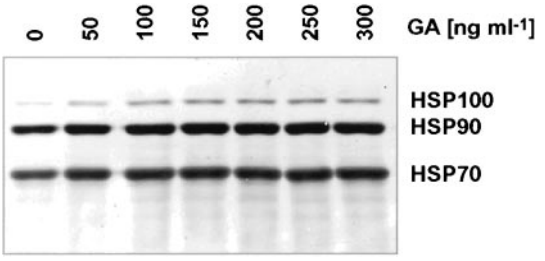

Figure 3. Effect of geldanamycin on Hsp levels in *Leishmania. L. donovani* promastigotes were incubated at increasing geldanamycin concentrations. After 24 h, cells were lysed, and the equivalent of 10^6 cells per lane was separated by SDS-PAGE. Proteins were blotted and probed with a mixture of antibodies directed against Hsp70, Hsp90, and Hsp100. Marker proteins (10-kDa ladder; Life Technologies) are shown on the left.

We first tested whether GA treatment can substitute for heat stress in the axenic amastigote development. As shown by immunoblot analysis (Figure 4A), promastigotes lack A2 proteins (lane 1). After 24 h treatment at 37°C, faint bands that represent the various A2 family members become visible (lane 2), the intensity of which increases considerably once the medium is acidified for 1–5 d (lane 3–7). Surprisingly, we detected A2 protein after cultivating *Leishmania* parasites for 24 h with geldanamycin at neutral pH and 25°C (lane 8). Further cultivation of the cells with geldanamycin in acidic milieu was sufficient to achieve A2 protein levels equivalent to standard stage differentiation conditions (lane 9–13).

To test the dosage effect, we exposed promastigotes to increasing GA concentrations. We also tested another specific Hsp90 inhibitor, RAD, and compared lysates of cells treated with these drugs for 2 d with lysates from heat-shocked and control cells, respectively (Figure 4B). Immunoblot analysis revealed that the induction of A2 protein synthesis started at the critical concentration of 25 ng/ml GA, whereas a lower dose of 5 ng/ml showed no effect. Hsp90 inhibition by RAD also induced A2 expression, albeit at a lower rate, whereas heat stress or the solvent DMSO had no such effect. Inhibition of Hsp90, therefore, is sufficient to mimic the environmental signals that induce the onset of A2 proteins synthesis.

In contrast, the escape populations that were kept cultivated under continuous GA exposure (100 ng/ml) showed only low levels of A2 proteins (lane 12) that barely increased when higher GA concentrations (lanes 13–19) or heat shock (lane 11) were applied. This is an indication that free Hsp90 is antagonistic to amastigote-specific gene expression.

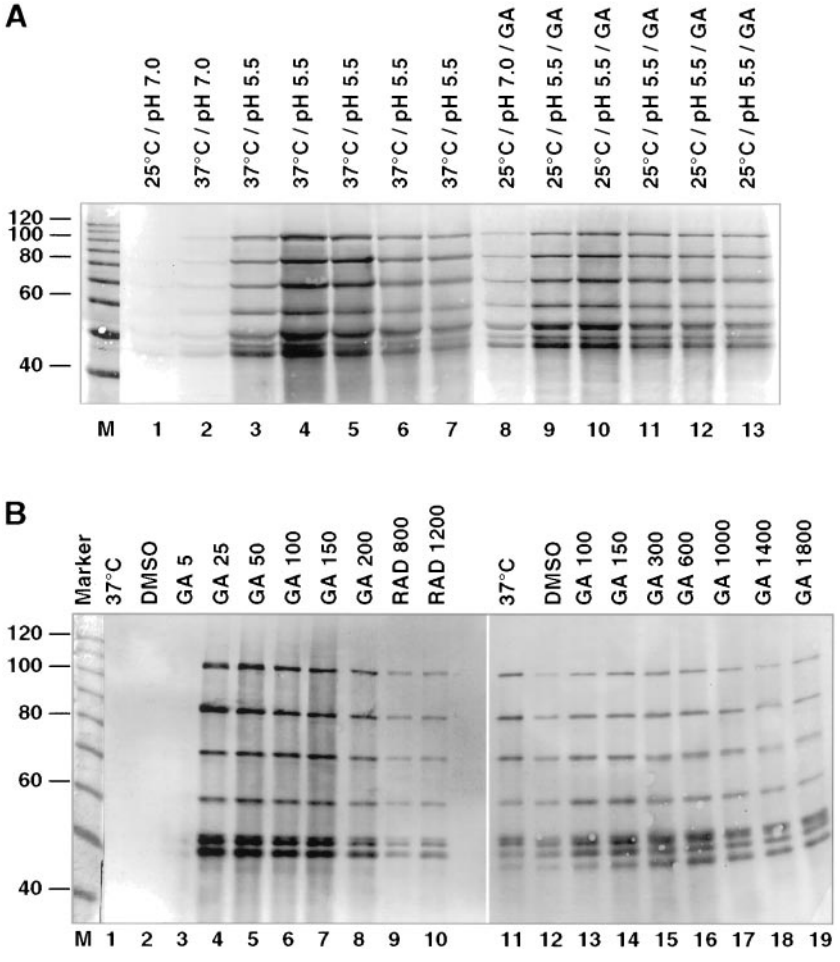

Figure 4. Geldanamycin-induced synthesis of amastigote-specific A2 proteins. (A) Immunoblot analysis. Samples were collected daily during an in vitro promastigote-to-amastigote differentiation of *L. donovani* and analyzed by SDS-PAGE and immunoblot with the use of anti-A2 mAb (lanes 1–7). Cells were treated with 200 ng/ml GA at 25°C (lane 8) for 24 h at neutral pH. The pH was shifted to 5.5, and incubation was continued at 25°C with 200 ng/ml GA (lanes 9–13). Note that a family of closely related proteins of different sizes is recognized by the antibody. (B) Immunoblot analysis of wild-type (lane 1–10) and escape strain I (lane 12–19) parasites, incubated for 48 h at 37°C (lanes 1 and 11), with the solvent DMSO (lanes 2 and 12), at the indicated GA concentrations (lanes 3–8 and 13–19), or at the indicated RAD concentrations (lanes 9 and 10). The equivalent of 10^6 cells per lane was subjected to SDS-PAGE and Western blot and probed with anti-A2 monoclonal antibodies. The positions of marker proteins (10-kDa ladder; Life Technologies) are shown on the left.

To exclude the possibility that a growth arrest per se will induce A2 protein synthesis, we tested various growth inhibitors at concentrations that had been found to block proliferation of promastigotes. Heat stress or stationary culture conditions did not induce A2 synthesis (Figure 5A). Neither did growth inhibition by hydroxy urea. Interestingly, taxol (Tx), which blocks the promastigote cell cycle in G2/M-phase, also induces A2 synthesis similar to RAD, but less strongly than GA.

It has been reported (Byrd *et al.*, 1999) that Hsp90 may interact directly with taxol. To test this possibility we compared A2 synthesis under taxol both in wild-type *L. donovani* and in the escape parasites that overexpress cytosolic Hsp90. As shown in Figure 5B, cells that overexpress Hsp90 require higher taxol concentrations to induce A2 protein synthesis. We therefore postulate that the induction by taxol is due at least in part to an interference of this drug with Hsp90 homeostasis.

Inactivation of Hsp90 Triggers Differentiation Toward the Amastigote

Typically, expression of the A2 proteins in *L. donovani* coincides with the morphological change associated with in vitro promastigote-to-amastigote differentiation. This change includes a length and size reduction and an almost complete loss of the flagellum that renders the amastigote nonmotile. To investigate whether inhibition of Hsp90 can induce a morphological differentiation, we analyzed, by indirect immunofluorescence, cells that had been cultivated under various growth conditions (Figure 6). Interestingly, a mere growth arrest by the G1 growth inhibitor hydroxy urea does not suffice to induce morphological change, and neither does acidification of the medium at 25°C. All three pharmacologicals that induce A2 protein synthesis and interact with Hsp90, GA, RAD, and Tx, induced a morphological differentiation similar to the developmental changes observed after exposure to heat stress and acidic pH.

To further analyze the changes induced by GA treatment, we compared promastigotes, axenic amastigotes, and GA-induced culture forms by scanning electron microscopy (Figure 7). The striking morphological changes during the differentiation from the promastigote (25°C, pH 7.0), during heat shock (37°C, pH 7.0), and after acidification (37°C, pH 5.5) are faithfully mimicked by an exposure to GA at neutral pH (25°C + GA, pH 7.0). We conclude that disturbances of Hsp90 homeostasis are a signal for the onset of stage differentiation.

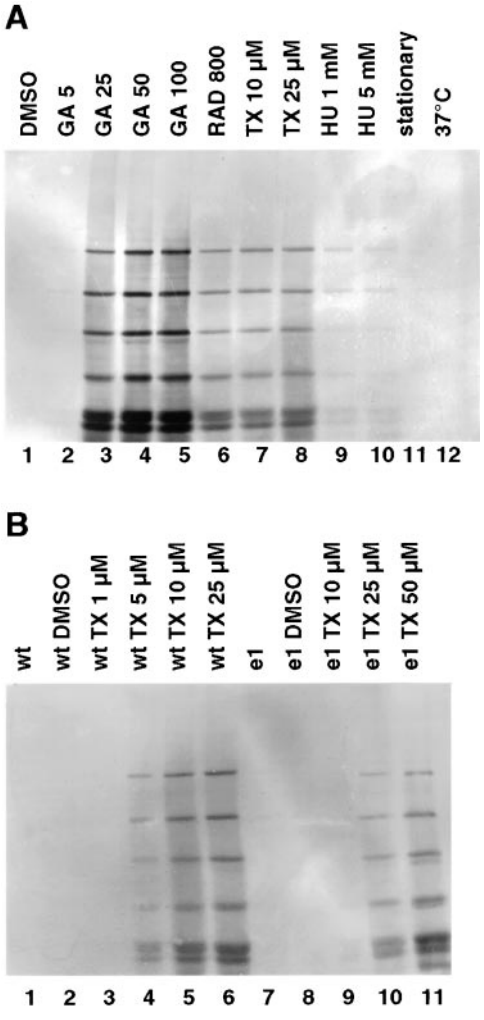

Figure 5. Taxol-induced synthesis of A2 protein family. (A) *Leishmania* promastigotes were incubated for 48 h at various concentrations (nanograms per milliliter) of geldanamycin (GA; lanes 2–5) or radicicol (RAD; lane 6). Alternatively, taxol (TX; lanes 7–8) and hydroxy urea (HU; lanes 9–10) were added at the indicated concentrations. Control cells were cultivated with the solvent DMSO (lane 1), until they were stationary (lane 11) or at 37°C (lane 12). (B) Wild-type (wt; lanes 1–6) and escape strain I (e1; lanes 7–11) promastigotes were incubated at the indicated concentrations of taxol (TX; lanes 4–6 and 9–11) or with the solvent DMSO (lanes 2 and 8). To exclude effects attributable to DMSO, in lane 1 and 7, lysates of untreated cells are shown. After 48 h the cells were lysed, and an equivalent of 10^6 cells per lane was subjected to SDS-PAGE and Western blot and probed with anti-A2 monoclonal antibodies.

DISCUSSION

What Is the Target of Geldanamycin?

Several lines of indirect evidence support the idea that the effects observed under geldanamycin or radicicol treatment are indeed due to an inhibition of the cytosolic form of Hsp90. 1) Limited overexpression of Hsp90 from episomal gene copies will raise the threshold concentration of GA-induced growth arrest. 2) Selection under GA produces escape populations that overexpress the cytosolic Hsp90. 3) Escape strains that overexpress Hsp90 require higher doses of GA or taxol to induce synthesis of amastigote markers. 4) A genetic complementation screen with the use of a shuttle cosmid DNA library and GA selection selected only for cosmids that harbor Hsp90 gene copies. DNA and amino acid sequences of Hsp90 and Grp94 are too far diverged to allow for cross-hybridization of DNA sequences or for cross-specificity of Hsp90-specific antibodies. Therefore, in all likelihood, the effects observed under geldanamycin treatment reflect an inhibition of the cytosolic Hsp90 species.

Cell Cycle Control and Hsp90

The known role of Hsp90 as a chaperone for cellular growth factors such as cyclin-dependent kinases, etc., may be an indication for the presence of similar regulatory factors in the leishmaniae. Hsp90 seems to contribute to the fast growth rates (three to four replication cycles per 24 h) that one can observe with cultured promastigotes. The amastigote stage, by comparison, shows only slow growth rates of one replication cycle per 48 h (Wiesgigl, unpublished observations). The sequestration of Hsp90 at elevated temperatures may be one determinant for the different growth rates of the two life cycle stages.

Implications of Rational Drug Design

The speedy appearance of spontaneous escape mutants after prolonged GA exposure is indicative of the inherent ability of *Leishmania spp.* to bypass targeted deactivation of important cellular proteins by episomal amplification of the respective genes and concomitant overexpression. This finding further illustrates a problem for efforts to develop drugs designed to target certain key proteins of these parasites ("rational drug design"). The import rates for such drugs must exceed the ability of the parasite to synthesize the respective target proteins de novo, even from highly amplified genes.

A Role for Hsp90 in Posttranscriptional Stress Response

In several eukaryotic systems, Hsp90 has emerged as the chaperone for proteins that mediate regulated transcription, cellular differentiation, development, and cell cycle control (Rutherford and Zuker, 1994; Scheibel and Buchner, 1998; Buchner, 1999), and it is part of the feedback regulation of the HSF-1-dependent stress response (Ali *et al.*, 1998; Zou *et al.*, 1998). In contrast to the model organisms investigated so far in this context, the kinetoplastida do not belong to the Eukaryote Crown Group. Comparative sequence analyses group them with the Euglenozoa, which must have branched off very early in the evolution of the eukaryota (Schlueter *et al.*, 2000). Nevertheless, as shown in this article, Hsp90 is used in a role very similar to its homologues in metazoan organisms. This implies that the function of Hsp90 is ancient. There is a remarkable difference, however, in that kinetoplastid protozoa do not use the major regulatory pathway common to all crown group eukaryotes, i.e., regulation of transcription mediated by transcription factors. Rather, transcription

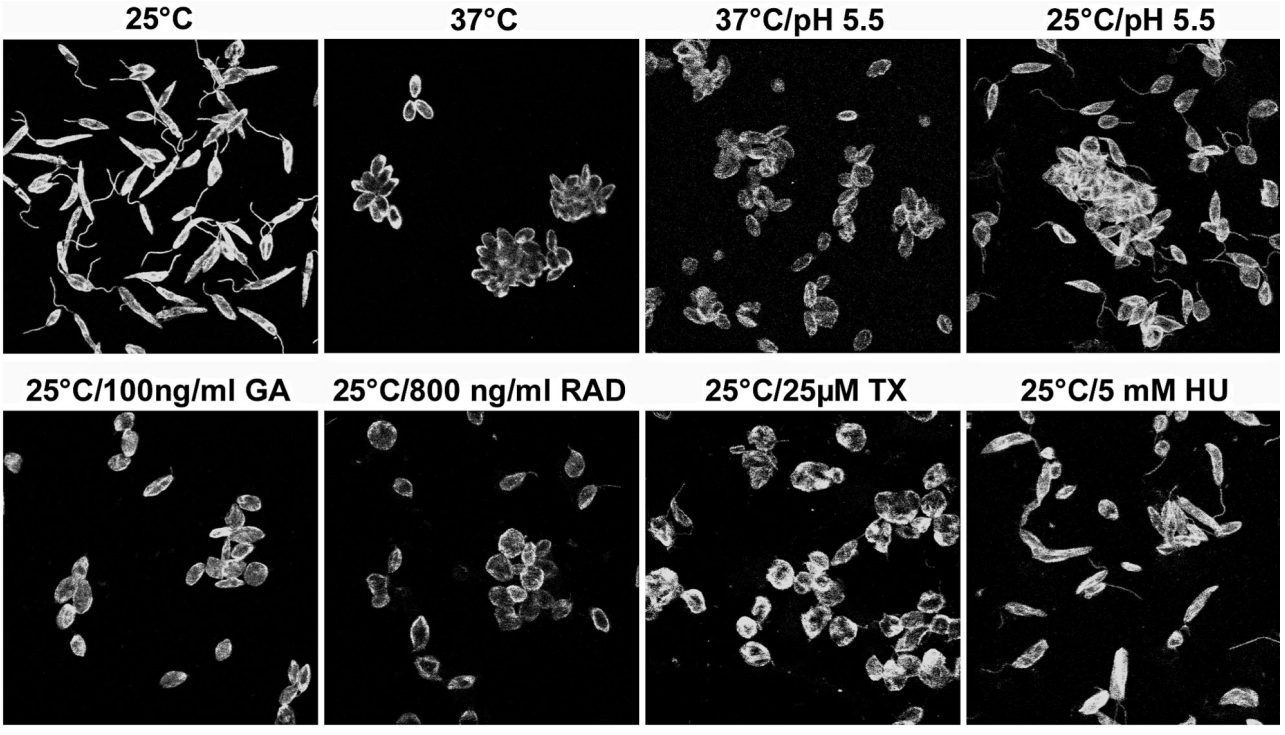

Figure 6. Morphological differentiation of *Leishmania* cells after Hsp90 inhibition. Promastigote parasites were cultivated for 48 h under the indicated conditions. Cells were then stained with anti–alpha-tubulin antibodies and anti-chicken IgG/DTAF and visualized by fluorescence microscopy.

probably proceeds unidirectionally (Myler *et al.*, 1999) and is mostly constitutive: gene expression is regulated at the levels of RNA processing, RNA stability, and/or translation control (Curotto de Lafaille *et al.*, 1992; Aly *et al.*, 1994; Brandau *et al.*, 1995; Graham, 1995; Teixeira, 1998; Stiles *et al.*, 1999). Nevertheless, Hsp90 can obviously chaperone the factors involved at these levels of regulation as well. It should be interesting in this context to investigate whether Hsp90 levels may play a role in the posttranscriptional regulation of Hsp synthesis in higher eukaryotes.

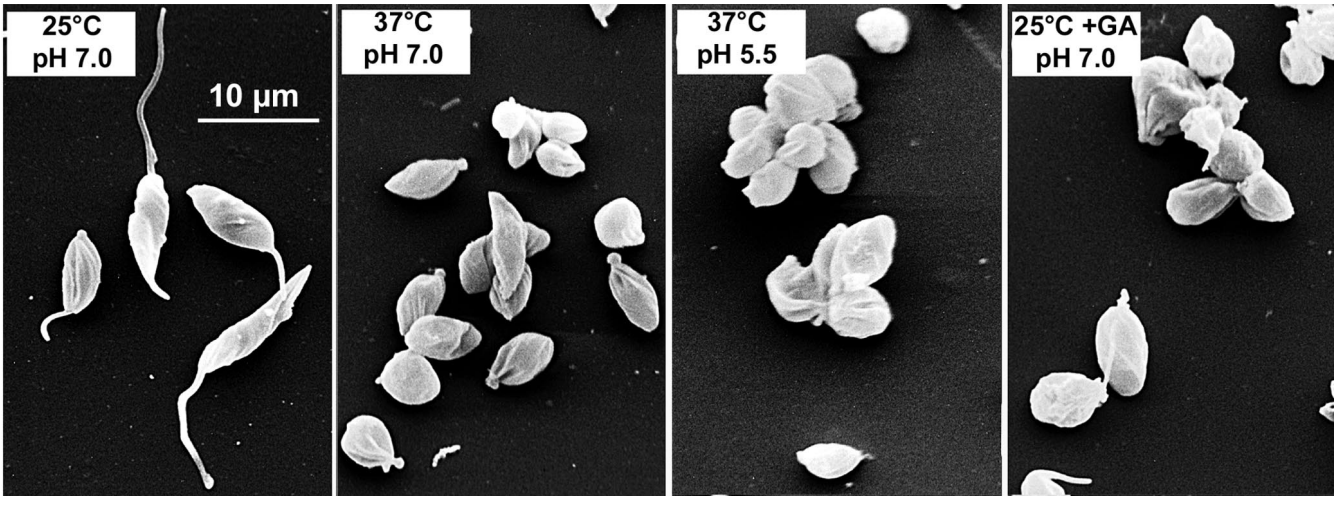

Figure 7. Morphological differentiation of GA-treated *Leishmania* cells. Promastigote *L. donovani* cells at 25°C, parasites incubated at 37°C for 24 h, axenic amastigotes after 5 d differentiation at 37°C and pH 5.5, and parasites treated at 25°C, 100 ng/ml GA for 24 h were imaged by scanning electron microscopy. Bar, 10 μm.

Antagonistic Roles of Hsp90 and Hsp100 in the Leishmania *Life Cycle*

The key outcome of our findings is the revelation of how a heat stress may be transduced into a developmental program, i.e., the differentiation from one life cycle stage to another. A sequestration of Hsp90 and Hsp70 by proteins that may denature at the elevated temperatures of a mammalian host can be envisioned. Indeed, Hsp90 has dual chaperone function. Although the Hsp90 multiprotein complex has specificity for cell cycle regulators and transcription factors, Hsp90 alone can act as a general chaperone stabilizing their structure of heat-labile proteins (Nathan *et al.*, 1997; Scheibel *et al.*, 1998). Under heat stress, Hsp90 complexes in *Leishmania* could therefore be in short supply to keep the cell cycle regulator proteins in a responsive state, thus abrogating their functions. In this, *Leishmania* may serve as a model for similar signaling pathways in other primitive eukaryotes.

So far, it is not clear whether GA-induced amastigote-like cells are identical or similar to axenic amastigotes cultivated under heat stress at acidic pH. In addition to morphological features and the expression of single marker genes, genome and proteome analysis should give more complete answers. The lack of transcription regulation in *Leishmania* and the incomplete genome project currently place severe limits on the use of genomics. The similarity of the proteome, on the other hand, should be a good indicator for comparing both culture forms, and this direction will be pursued. Differences, if any, should yield valuable information because they may allow a distinction between separate regulatory pathways, those including Hsp90 and others that may be independent of this chaperone.

If inactivation of Hsp90 is a signal for the induction of stress response, growth arrest, and differentiation toward the amastigote, how is the amastigote stage maintained? We know that induced synthesis of Hsp90 and Hsp70 ceases after a few hours of heat stress (Wiesgigl, unpublished observations). This would indicate that the system somehow approaches homeostasis; here is where a role of Hsp100 may lie. This chaperone is synthesized continuously under heat stress and reaches high concentrations in amastigotes of both *L. donovani* and *L. major* (Hubel *et al.*, 1995, 1997; Krobitsch *et al.*, 1998). We know that amastigote-to-promastigote differentiation and the concomitant resumption of rapid cell divisions is significantly accelerated in Δ*clpB* mutants of *L. donovani* that lack Hsp100 (Krobitsch and Clos, 1999). Once synthesized to relevant concentrations, Hsp100 may stabilize the amastigote stage by antagonizing promastigote development. The idea of antagonistic functions of Hsp90 and Hsp100 draws support from our finding that expression of the amastigote-specific A2 protein family, normally impaired by the lack of Hsp100 in Δ*clpB* mutants of *L. donovani*, can be restored by the pharmacological inactivation of Hsp90 (Wiesgigl, unpublished observations). It thus seems that heat shock proteins, by their balanced functions as chaperones, can establish regulatory pathways in primitive eukaryota.

CONCLUSION

In the case of the leishmaniae, the stress response appears to be used by the parasites to sense their respective environment, fly gut, or mammalian macrophage, and to trigger the appropriate developmental program. It will be of interest to test whether similar regulatory functions can be shown for the Hsp90 of other primitive eukaryotes, either parasitic or free-living.

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